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HYBRIDOMA, vol. 6, no. 3, March 1987, New
York, US; A. MAEGER et al., pp.
305-312&NUM;

HYBRIDOMA, vol. 6, no. 4, April 1987, New
York, US; B.M. FENDLEY et al., pp. 359-370;

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CLINICAL RESEARCH, vol. 35, no. 1, January 1987, THOROFARE US; A.A. CREASY et al., p. 142a;

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 137, no. 2, 13 June 1986, New York, US; C.-M. LIANG et al., pp. 847-854;

SCANDINAVIAN JOURNAL OF IMMUNOLOGY, vol. 24, no. 6, 1986, Oslo, Norway; A. WAAGE et al., pp. 739-743;

CHEMICAL ABSTRACTS, vol. 106, no. 7, 16 February 1987, Columbus, Ohio, US; D.Y.M. LEUNG et al., pp. 1958-1972, abstract no. 48416z;

IMMUNOBIOLOGY, vol. 175, no. 1,2, Stuttgart DE, A. MEAGER et al., p. 83;

IMMUNOBIOLOGY, vol. 175, no. 1,2, 1987, Stuttgart DE; K. YONE et al., p. 106;

CLINICAL CHEMISTRY, vol. 33, no. 11, November 1987, Winston, US; A.M. TEPPPO et al., pp. 2024-2027;

NATURE, vol. 312, 20 December 1984, London, GB; D. PENNICA et al., pp. 724-729;

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D scription

This invention relates to a method for confirming a diagnosis of Kawasaki disease in a patient and a detection kit for determining the diseased condition of a subject.

More specifically, this method comprises detecting tumor necrosis factor (TNF hereinafter) in a body fluid of a subject by using an antibody specific for TNF and determining the progress of the diseased condition of the subject.

TNF was discovered as a physiologically active substance which appears in the blood in a CD-1 Swiss mouse when the mouse is given Bacillus Calmette-Guerin (BCG) and 2 weeks later, endotoxin is administered to the mouse. It was reported by Carswell in 1975 [E. A. Carswell et al., Proc. Natl. Acad. Sci., U. S. A., 72, 3666 (1975)]. The amino acid sequence of this biologically active protein was determined by Aggarwal et al. in 1985 [B. B. Aggarwal et al., J. Biol. Chem. 260, 2345 (1985)].

Pennica et al., Shirai et al. and Wang et al. determined the amino acid sequence and the DNA sequence of human TNF [Pennica et al., Nature 312, 724 (1985); Shirai et al., Nature 313, 803 (1985), and Wang et al., Science 228, 149 (1985)]. Initially, TNF was exploited as a cancer therapeutic agent because of its antitumor activity. Recently its various physiological activities were elucidated, and its various functions *in vivo* have been investigated. For example, TNF has the activity of a mediator *in vivo* in endotoxin shock induced by bacterial infection [B. Beutler et al., Science, 229, 869 (1985)], the activity of inducing an inflammatory reaction on vascular endothelial cells [J. R. Gamble et al., Proc. Natl. Acad. Sci., U. S. A. 82, 8667 (1985), and pyrogenic activity [C. A. Dinarello et al., J. Exp. Med. 163, 14443, 1363, (1986)], and the activity of inducing another inflammatory cytokine, interleukin 1, one inflammation-causing substance [P. P. Nawroth et al., J. Exp. Med., 163, 1363 (1986)] or prostaglandins [P. R. Backwich et al., Biochem. Biophys. Res. Comm., 136, 94 (1986)].

In CLINICAL RESEARCH (1987), vol. 35, no. 1, page 142a; A. Creasy et al, "Murine monoclonal antibodies define neutralizing epitopes on human tumor necrosis factor" a panel of four monoclonal antibodies (MAbs) have been raised against human recombinant tumor necrosis factor (rTNF). These MAbs have been characterized for their ability to immunoprecipitate ¹²⁵I-labelled rTNF, block binding of ¹²⁵I-labelled rTNF to L929 mouse fibroblasts and to neutralize *in vitro* cytotoxicity of rTNF and native TNF (nTNF) in the L929 cytotoxicity assay.

In Chemical Abstracts (1987), vol. 106, no. 48416z; D. Leung et al, "Two monokines, interleukin 1 and TNF, render cultured vascular endothelial cells susceptible to lysis by antibodies circulating during Kawasaki syndrome", it is only mentioned that there are cytotoxic IgG and IgM present in the blood of a patient of Kawasaki disease, and that, when vascular endothelial cells are stimulated by interleukin 1 (IL-1) or TNF, a certain kind of antibody is formed on the surface of the vascular endothelial cells, to which antibody the IgG and IgM bond, causing a disorder in the vascular endothelial cells. Moreover, the above opinion is derived from an experiment *in vitro*.

This document describes that, firstly, a monocyte secretes IL-1 or TNF, which causes a disorder and a change on the surface of vascular endothelial cells, and it is not suggested at all that TNF is present at a high level in the blood of a patient of Kawasaki disease.

HYBRIDOMA (1987) vol. 6, no. 3, pages 305 to 312; A. Meager et al, "Preparation of monoclonal antibodies directed against antigenic determinants of recombinant human TNF" is directed to the preparation and characterization of monoclonal antibodies directed against antigenic determinants of recombinant human tumor necrosis factor (rTNF). This document is completely silent as to the diagnosis of a patient of Kawasaki disease.

HYBRIDOMA (1987), vol. 6, no. 4, pages 359 to 370; B. Fendley et al, "Murine monoclonal antibodies define neutralizing epitopes on human tumor necrosis factor" discloses a panel of four monoclonal antibodies (MAbs) against recombinant human tumor necrosis factor- α (rTNF). These MAbs immunoprecipitate ¹²⁵I-labelled rTNF, block binding of ¹²⁵I-labelled rTNF to L929 mouse fibroblasts, and neutralize *in vitro* cytotoxicity of rTNF and native TNF (nTNF) in the L929 cytotoxicity assay.

BIOCHEM. BIOPHYS. RES. COMMS (1986), vol. 137, no. 2, pages 847 to 854; Chi-Ming Liang et al, "Production and characterization of monoclonal antibodies against recombinant human TNF/cachectin" discloses that the tumor necrosis factor is a monokine which causes cytolysis of many transformed cells. In this study it was found that in addition to cytotoxicity recombinant *Escherichia coli*-derived human tumor necrosis factor, like cachectin, inhibited the lipoprotein lipase of 3T3-L1 preadipocytes. Both effects were inhibited by monoclonal anti-tumor necrosis factor antibodies.

In view of such many research results, TNF is considered to have hormone-like activity as an information transmitting substance *in vivo* which provokes many physiological actions. These research results therefore suggest a possibility that quantitative changes of TNF *in vitro* such as abnormal increases

or decreases may have to do with the conditions of many diseases. The present inventors thus presumed that detection of the presence of TNF in a body fluid such as blood and measurement of its concentration would be beneficial for monitoring many diseases.

Investigations of the present inventors have shown that the body fluid of a patient contains TNF and the amount of TNF has closely to do with the progress of his diseased condition; that, therefore, a change of his diseased condition, especially aggravation, can be predicted to some extent by accurately determining the concentration of TNF in the body fluid and measuring its quantitative changes; and that by performing a preventive or curative treatment before such a change or aggravation occurs, the progress of the patient's disease can be hampered and sometimes, the disease can be cured.

It has been found particularly by the investigations of the present inventors that the body fluids of patients of Kawasaki disease whose diagnosis and progress are extremely difficult to check and patients of bacterial infections contain TNF in larger quantities than those of normal healthy persons, and the concentration of TNF and its variations have closely to do with the progress of these diseases. It is very important therefore in checking the progress of the diseases of such patients to measure the concentration of TNF in the body fluids accurately and rapidly.

Kawasaki disease (infantile acute febrile mucocutaneous lymphnode syndrome: MCLS), which was reported by Kawasaki in 1967 [T. Kawasaki, "Allergy", 16, 178 (1967)], is a disease of unknown etiology which most frequently affects infants and children under 5 years of age. The disease is mainly characterized by prolonged fever (5 days or longer), reddening and indurative edema of hands and feet, membranous desquamation from finger tips during the convalescent stage of the disease, changes in lips and oral cavity (reddening of lips, strawberry tongue, diffuse injection of oral and pharyngeal mucosa), polymorphous exanthema, bilateral conjunctival congestion, and acute nonpurulent cervical lymphadenopathy.

Kawasaki disease has been diagnosed on the standards disclosed in T. Kawasaki, "Shonica" (Pediatrics), 26, 935 (1985). It is very difficult to diagnose definitely because its etiology is unknown and it is diagnosed as a combination of some principal conditions. In particular, the formation of coronary aneurysm in this disease is a main cause of death. It is an urgent and important problem to find out an index of the diseased condition of Kawasaki disease which may lead to prediction of the formation of such coronary aneurysm with the progress of the disease.

The present inventors have found that in patients of Kawasaki disease of a relatively serious condition which may lead to the formation of coronary aneurysm, TNF is markedly detected from the patients' body fluid (e.g., serum).

The present inventors have also found that from a body fluid (e.g., sera) of patients of bacterial infections, TNF is detected in good correspondence to changes in the amounts of white blood cells, platelets and C reactive proteins in it, and therefore that it is very significant for the diagnosis of the condition of a bacterial infection and determination of the progress of its diseased condition to measure TNF in a body fluid.

Thus, according to this invention, there is provided a method for confirming a diagnosis of Kawasaki disease in a patient which comprises

contacting an anti-human tumor necrosis factor antibody with a sample of body fluid from the patient, detecting an amount of a substance which specifically binds the anti-human tumor necrosis factor antibody, and

comparing the amount of the substance with that in a sample of body fluid taken from a normal, healthy person

wherein elevated levels of the substance are indicative of Kawasaki disease.

The present invention is used to determine the diseased conditions of patients of Kawasaki diseases.

The body fluid to be taken from the subject may be any liquid component which exists in a human body, and is especially desirably a blood component such as serum or plasma because of its ease of utilization. It may also be an exudate from an inflammatory site, lymph or a joint effusion or a blood component of a diseased region or its neighborhood. When the change of the amount of TNF is to be examined by measuring it a number of times, it is desirable to utilize the blood component of the peripheral blood taken by an ordinary method. But this is not limitative.

An anti-TNF antibody is used to measure the substance reactive with anti-TNF antibody in the body fluid of the subject by immunological assay. An anti-serum or monoclonal antibody specific for TNF is used as the antibody. The known sandwich method is advantageously used for the immunological assay. Usually, two types of antibodies are used in the sandwich method. In the detection method of this invention, too, the anti-TNF monoclonal antibody, particularly an anti-human TNF monoclonal antibody, is preferably used as one of the two antibodies because it permits accurate and rapid detection of TNF.

The sandwich method advantageously used in the immunological assay in this invention is a known method which is frequently used to determine the very low concentration of a specific protein using antibodies, and is described, for example, in Langone, J. J. and van Vunakis, H. (eds.): *Methods in Enzymology*, Vol. 73, Immunochemical Techniques, Part B, Academic Press, New York, 1981; Nakamura, R. M., Ioto, W. R. and Tucker, E. S., III (eds.): *Immunoassays in the Clinical Laboratory*, Alan R. Liss, New York, 1979; and Ishikawa, E., Kawai, T. and Miyai, K. (eds.): *Enzyme Immunoassay*, Igaku-Shoin, Tokyo, 1981.

The Kawasaki disease or a condition suspected of it, which is to be detected in accordance with this invention, is a disease or condition in which a substance reactive with an anti-TNF, specifically TNF, is contained in the body fluid in an amount larger than that in a normal healthy person.

The content of TNF or its changes in the body fluid of subject constitute an important standard of determining the diseased condition.

The present invention provides a method for accurate and rapid detection of an anti-TNF antibody-reactive substance in a body fluid taken from a subject using an anti-TNF antibody by immunological assay, and a kit therefor.

To detect the amount of TNF in the subject accurately is necessary for accurate determination of the diseased condition of the subject. If the detection is delayed, it is likely that the time for applying the results of the detection to the hampering of progress of the disease or to the treatment of the disease will be retarded or lost. One type of the anti-human TNF monoclonal antibody is described in Japanese Laid-Open Patent Publication No. 208924/1985. The monoclonal antibody described in this patent document binds to human TNF and inhibits its activity. The patent document, however, fails to describe to what epitope of human TNF it binds.

It has been found in accordance with this invention that the use of an anti-human TNF monoclonal antibody having the following characteristics (a) to (c).

(a) It has the ability to neutralize the cytotoxic effect of human TNF on L929 cells and the inhibitory effect of human TNF on fatty acid metabolism.

(b) It recognizes epitopes contained in the 68th (Gly) to the 97th (Ile) amino acids in the amino acid sequence of human TNF.

(c) It specifically inhibits binding of human TNF to a TNF receptor.

To the best of the knowledge of the present inventors, the anti-human TNF monoclonal antibody having the characteristics (a) to (c) is novel, and has been provided for the first time by the present invention (this monoclonal antibody is abbreviated as "MCA-A" hereinafter).

According to this invention, there is described an anti-human TNF monoclonal antibody which recognizes epitopes contained in the 7th (Thr) to the 37th (Leu) amino acids in the amino acid sequence of human TNF (this antibody will be abbreviated as "MCA-B"). MCA-B does not neutralize the cytotoxic effect of human TNF on L929 cells or its effect of inhibiting fatty acid metabolism.

According to this invention, there is further described an anti-human TNF monoclonal antibody which recognizes epitopes contained in the 113th (Pro) to the 127th (Glu) amino acids in the amino acid sequence of human TNF (this antibody will sometimes be abbreviated as "MCA-C"). MCA-C neither neutralizes the cytotoxic effect of human TNF on L929 cells nor its effect of inhibiting fatty acid metabolism. It neither inhibits binding of human TNF to a TNF receptor.

MCA-A, MCA-B and MCA-C are all novel monoclonal antibodies to human TNF, and can be used as at least one anti-TNF antibody in the detection method and the detection kit provided by this invention. MCA-A is especially preferably used in view of its binding activity.

Now, the preparation of the above monoclonal antibodies to human TNF and the detection kit will be described in detail.

Preparation of anti-TNF antibody

(1) Preparation of cells for producing anti-TNF antibody

Antibody-producing cells are prepared in accordance with a conventional method by immunizing an animal with human TNF as an antigen and recovering the antibody-producing cells from the animal. Suitable animals for use in this method include mice, rats, rabbits, guinea pigs, goats, horses and bovine. The antibody-producing cells may be, for example, spleen cells, lymph node cells and peripheral blood cells.

(2) Preparation of myeloma cells

There is no particular restriction on the myeloma cells used in cell fusion, and cell lines from many animals such as mice, rats, rabbits and humans can be used. Preferably, the cell lines used should have drug resistance, and be such that the unfused myeloma cells cannot survive in a selective medium and only hybridoma cells can proliferate in it. A cell line having resistance to 8-azaguanine is most commonly used. It lacks hypoxanthine-guanine-phosphoribosyl transferase and cannot grow in HAT medium (hypoxanthine-aminopterin-thymidine). Preferably, the cell lines used are of the "non-secreting type". Preferably used are, for example, P₃/X63-Ag8U1(P₃U1), P₃/X63-Ag.6.5.3, P₃/NS1-1-Ag4-1 and Sp2/O-Ag14 derived from murine myeloma cell line MOPC-21, and rat myeloma cells 210 RCY3.Ag1.2.3.

(3) Cell fusion

Usually, 1 to 5x10⁷ myeloma cells and 1 to 5x10⁶ antibody-producing cells are mixed (usually in a ratio of from 1:4 to 1:10) in a culture medium, usually Eagle's minimum essential medium (MEM) or Rosewell Park Memorial Institute (RPMI)-1640 medium, and fused in the presence of a fusion promoter preferably polyethylene glycol (PEG) having an average molecular weight of 1000 to 6000. The concentration of PEG used is usually 30 to 50 %. Viruses may also be used as the fusion promoter.

(4) Selective proliferation of hybridoma cells

After the cell fusion, the cells are suitably diluted with, for example, RPMI 1640 medium containing 10 % bovine fetal serum, and plated on a microtiter plate at a rate of about 10⁵ cells per well. A selective medium (such as HAT medium) is added to each well, and the cells are cultivated while properly replacing the selective medium with a fresh one. If a 8-azaguanine-resistant strain is used as the myeloma cells, all unfused myeloma cells will die in about 10 days in the HAT medium. Furthermore, since the antibody-producing cells are normal cells, they cannot survive for a long time in vitro. Accordingly, cells which grow after 10 to 14 days from the start of cultivation are hybridoma cells.

(5) Screening of antibody-producing hybridoma cells

Screening of the hybridoma cells may be carried out in a customary manner. For example, part of the supernatant in the well in which the hybridoma cells have grown is sampled and reacted with human TNF or fixed human TNF, and then by reaction with a second antibody labelled with, for example, an enzyme, a radioisotope, a fluorescent substance, or a light-emitting substance, the amount of labelling is measured. Thus, the presence of an anti-human TNF antibody can be assayed.

(6) Cloning

There is a possibility that two or more clone of hybridoma cells will grow in each well. Hence, cloning is carried out, for example, by a limiting dilution method and hybridoma cells producing a monoclonal antibody are obtained.

(7) Isolation of the antibody

The purest monoclonal antibody can be obtained from the supernatant obtained by cultivating the hybridoma cells in a suitable culture medium such as RPMI-1640 medium containing about 10 % of bovine fetal serum.

To obtain a large quantity of the antibody, a mineral oil such as pristane (2,6,10,14-tetramethylpentadecane) is intraperitoneally administered to an animal which is the same strain as the animal from which the myeloma cells have been taken. Thereafter, the hybridoma cells are administered to the animal and grown in large quantities in vivo. Ascites tumor is formed in 10 to 18 days after the administration, and the antibody is formed in a high concentration in the serum and ascites.

Kit for detection of human TNF and preparation thereof

The monoclonal antibodies to human TNF obtained as above are utilized as at least a part of the anti-TNF antibodies used in the detection method and detection kit of the invention.

In the immunological assay, especially the sandwich method, two types of antibodies which can bind to the target antigen (e.g., protein) are generally used. In the present invention, two types of antibodies are likewise used, and the above monoclonal antibody specific for human TNF is used as at least one of the two antibodies. The two antibodies may both be monoclonal antibodies which recognize different epitopes of human TNF. Alternatively, one of the antibodies may be a monoclonal antibody to human TNF, and the other, an antiserum to human TNF.

A kit comprising two antibodies to human TNF will now be described.

An antibody (first antibody) to human TNF is fixed to a suitable insoluble solid carrier (such as a plastic container). The antibody so fixed will be referred to as the fixed antibody" hereinafter. The surface of the insoluble solid carrier is then coated with a suitable substance (such as bovine serum albumin) in order to avoid a non-specific binding of an assay sample (e.g., serum) to be assayed.

The insoluble solid carrier to which the first antibody is fixed is contacted, and reacted, with the assay sample at a predetermined temperature for a predetermined period of time. During this time, the fixed antibody (first antibody) binds to TNF in the assay sample. The fixed antibody is then washed with a suitable washing solution, and then a solution (e.g., aqueous solution) of an antibody (second antibody) to human TNF which antibody is labelled with a suitable labelling substance is contacted, and reacted, with TNF bound to the fixed antibody in the insoluble solid carrier at a predetermined temperature for a predetermined period of time. The carrier is washed with a suitable washing solution, and then the amount of the labelling substance which exists on the insoluble solid carrier is measured. From the measured amount, the amount of TNF in the assay sample can be calculated.

Thus, the assay kit of this invention is composed mainly of a fixed antibody obtaining by binding a first antibody to an insoluble solid carrier and a labelled second antibody. To utilize this kit efficiently, conveniently and simply, the kit is constructed by incorporating various supplements besides the antibodies. The supplements include, for example a dissolving agent for dissolving a solid reagent, a washing agent for washing the insoluble solid carrier, a substrate for measuring enzyme activity when an enzyme is used as the labelling substance for the antibody, and a reaction stopper. These supplements are normally used in kits of immunological assay reagents.

Examples of the insoluble solid carrier used in the assay kit of this invention are polymers such as polystyrene, polyethylene, polypropylene, polyesters, polyacrylonitrile, fluorine resins, crosslinked dextran and polysaccharides, paper, glass, metal, agarose and combinations of these.

The insoluble solid carrier may be in the form of, for example, a tray, a sphere, a fiber, a rod, a disc, a receptacle, a cell or a test tube.

Radioactive substances, enzymes or fluorescent substances are advantageously used as the labelling substances. Examples of the radioactive substances are ^{125}I , ^{131}I , ^{14}C and ^3H . Examples of the enzymes are alkaline phosphatase, peroxidase and beta-D-galactosidase. Examples of the fluorescent substances include fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate. These are not limitative, and other labelling substances which can be applied to immunological assay may also be used. Adjuvants may also be used to increase the sensitivity of the labelling substances.

In the detection method and kit of this invention, the aforesaid anti-TNF antibodies are used as the first and second antibodies. Specifically, two types of monoclonal antibodies which will give good results in a quantitative assay system are selected from the monoclonal antibodies produced by the method described hereinabove, and used as the first and second antibodies. An anti-human TNF antiserum obtained in a customary manner from a mammal may be used as one of the first and second antibodies.

Brief Description of the Accompanying Drawings

Figure 1 shows the binding activity of an anti-TNF monoclonal antibody to TNF;

Figure 2 shows the inhibiting effect of synthetic peptides D (see Table 2) on the binding of monoclonal antibody 1F12A7 to TNF;

Figure 3 is a calibration curve for assaying human TNF;

Figure 4 shows the results of measurement of the amounts of TNF in a serum sample of a patient of Kawasaki disease and a healthy human subject (TNF);

Figure 5 shows the relation between the serum TNF level and the appearance of coronary involvement; and

Figure 6 shows the fever pattern, change of the number of white blood cells (WBC), of the number of platelets (PLT) and of the TNF content in a patient with Kawasaki disease and the appearance of a diseased change in coronary artery.

The following examples illustrate the present invention in greater detail. It should be understood however that the invention is not limited by these examples.

EXAMPLE 1

Preparation of an anti-human TNF monoclonal antibody:-

Purification of an antigen

Human TNF used in this invention was prepared by the method described in the earlier application, Japanese Patent Application No. 90087/1986 filed on April 21, 1986.

Specifically, *Escherichia coli* into which a vector expressing human TNF DNA had been introduced was cultivated to induce production of human TNF protein. After harvesting, the *E. coli* cells were ruptured by ultrasonication. The resulting suspension was purified by column chromatography on DEAE-Sepharose by the method of Shirai et al. [T. Shirai, Nature, 313, 830 (1985)]. The coarsely purified product contained about 30 % of TNF.

Immunization of a mouse with human TNF

An emulsion of the above TNF fraction (50 to 100 micrograms) formed with complete Freund's adjuvant was administered to male Balb/c mice at 2-week intervals. Four days after the final immunization, the spleen was taken out for cell fusion.

Cell fusion

Cell fusion was carried out in a customary manner. Using a mesh, a cell suspension was prepared from the spleen aseptically taken out from the immunized animal. The cell suspension was washed three times with RPMI-1640 medium, and then mixed with murine myeloma cells, P3/X63-Ag8U1 (P3U1 for short) [see D. E. Yelton et al., Current Topics in Microbiology and Immunology, 81, 1 (1978)] in a mixing ratio of from about 1:1 to 5:1, and then centrifuged. 1 ml of an RPMI-1640 solution of 50 % polyethylene glycol 1540 was gradually added. The centrifuge tube was slowly stirred for 1 minute to effect cell fusion. Furthermore, RPMI-1640 medium was gradually added over an extended period of time to a final volume of 10 ml. After centrifugation, the pellets were suspended in RPMI-1640 medium containing 10 % bovine fetal serum so that the number of the myeloma cells was 5 to 10×10^4 cells/0.1 ml. The suspension was plated on a 96-well microtiter plate (Costar) at a rate of 0.1 ml per well.

One day later, HAT medium was added at a rate of 0.1 ml per well, and thereafter, half of the medium was replaced by HAT medium at suitable time (day) intervals. On the 5th day and onward the growth of hybridoma cells was observed in some wells, and in two weeks, the hybridoma cells proliferated in almost all wells.

Screening and cloning of antibody-producing cells

0.1 ml of the culture supernatant was taken from the well where the hybridoma cells grew, and incubated with human TNF fixed to an immunoassay plate (Titertek), and the antibody which bound to human TNF were detected. With a probability of about 40 %, there were observed wells in which antibodies having binding activity to human TNF were secreted. Only those hybridoma cells which secreted antibodies having high binding activity to human TNF were selected, plated at a density of one cell per well, and cloned by the limiting dilution method. From the clones obtained, 93 clones were selected and cultivated in RPMI-1640 containing 10 % bovine fetal serum while increasing the scale from a 96-well plate, a 24-well plate, 6-well plate and finally 25 cm² flask. The culture supernatants were collected.

Each of the supernatants were mixed with human TNF solution (1000 units/ml) and incubated at 37 °C for 1 hour and then subjected to evaluation of TNF activity using L929 cells. Eleven clones having the high ability to neutralize TNF activity (9C4G5, 8E6B6, 10B7E11, 11D7G4, 8E6C6, 2B2H10, 9C4A9, 8E6D7, 1G7D3, 8E6G4, 10B7C6) and 5 clones having the high ability to bind to TNF but hardly any ability to neutralize TNF activity (9E8G7, 1A10D4, 1F12A7, 1F12A9, P4B4D5) were selected.

Purification of monoclonal antibodies

The above 16 clones were further cultivated on an increased scale using RPMI-1640 medium containing 10 % bovine fetal serum, and about 500 ml to 10 liters of the culture supernatants were collected. The supernatants were stirred with 50 % saturated ammonium sulfate at 4 °C for about 1 hour, and then centrifuged at 10,000G for 30 minutes. The pellets were dissolved in a small amount of deionized water and dialyzed against 0.1M phosphate buffer (pH 8.0). The solution was charged on a protein A Sepharose (Pharmacia) column and the adsorbed monoclonal antibodies were eluted with 0.1M citrate buffer (pH 5.0 or 3.0). The eluates were neutralized with sodium hydroxide and then concentrated by a membrane filter (Amicon YM-10). The buffer was replaced by 0.1M phosphate buffer (pH 8.0) to form solutions of purified monoclonal antibodies.

EXAMPLE 2

15 Determination of the ability to specifically recognize TNF:-

The above coarsely purified TNF and purified TNF were electrophoresed on SDS-PAGE PLATE 10/20 (Daiichi Chemical Co., Ltd.), and then blotted on a nitrocellulose filter in a blotting buffer (20 mM Tris, 150 mM glycine and 20% methanol). The nitrocellulose filter was shaken at room temperature for 1 to 2 hours in a buffer (20 mM Tris, 500 mM NaCl) containing 3 % gelatin. The buffer was then replaced by a buffer containing 2 micrograms/ml of the purified antibody and 1 % gelatin, and the nitrocellulose filter was shaken overnight at room temperature. The nitrocellulose filter was washed three times with a washing solution (20 mM Tris, 500 mM NaCl and 0.05 % Tween 20). Goat anti-mouse IgG-HRP complex (Bio-Rad Company) was diluted with a buffer containing 1 % gelatin to the dilution ratio specified by the manufacturer, and the nitrocellulose filter was shaken in this solution at room temperature for 1 to 2 hours, then washed three times with the aforesaid washing solution and then with a washing solution not containing Tween 20, and then colored with HRP Development Reagent (Bio-Rad Company). The results obtained by using 1F12A7 as the purified antibody are shown in Figure 1. It is clearly seen from this figure that 1F12A7 bound both to the coarsely purified TNF and the purified TNF and thus has the ability to specifically binding to TNF.

30 By the same procedure, it was confirmed that antibodies 9C4G5, 8E6B6, 10B7E11, 11D7G4, 8E6C6, 2B2H10, 9C4A9, 8E6D7, 1G7D3, 8E6G4, 10B7C6, 9E8G7, 1A10D4, 1F12A9 and P4B4D5 have the ability to specifically binding to TNF.

EXAMPLE 3

35 Study of the ability to neutralize the TNF's effect of inhibiting fatty acid metabolism:-

Mouse fibroblast cells were confluent and then subjected to induction of differentiation for two days in LMDMEM medium containing 10 % bovine fetal serum and 115 micrograms/ml of MIX (3-isobutyl-methyl-xanthine), 395 ng/ml of DEX (dexamethasone) and 10 micrograms/ml of INS (bovine insulin) to differentiate the cells into fat cells. Two days after the induction, the culture medium was replaced by a differentiation maintaining medium (LMDMEM medium containing 10 % bovine fetal serum and 50 ng/ml of INS), and a sample was added 1 to 2 hours later. The sample was TNF added to attain a final concentration of 47 ng/ml, or a product obtained by mixing TNF and an anti-TNF antibody in a molar ratio of 1:1 and incubating the mixture at room temperature for 1 hour which was added so as to attain a TNF concentration of 47 ng/ml. After adding the sample, the mixture was incubated at 37 °C for 4 hours, and then heparin was added so as to attain a final concentration of 10U/ml. The incubation was carried out further for 1 hour. After the incubation, the supernatant was taken out, and the lipoprotein lipase activity in the supernatant was measured by reacting it with ³H-labelled triolein as a substrate. The results are shown in Table 1 below. It is evident from Table 1 that the anti-TNF antibody which can neutralize the cytotoxic effect of TNF on L929 also neutralize the action of TNF to inhibit fatty acid metabolism of 3T3-L1 fat cells. It is also clearly seen from the table that the anti-TNF antibodies which do not neutralize the cytotoxic effect of TNF do not neutralize the action of TNF to inhibit fatty acid metabolism of 3T3-L1 fat cells.

Table 1

	Antibody	Ability of neutralizing inhibition of LPL activity (%)
Neutralizing antibody	11D7G4	100
	8E6G4	67
	9C4A9	31
	1G7D3	34
Non-neutralizing antibody	1A10D4	0
	1F12A7	0
	1F12A9	0
	9E8G7	0
	Mouse IgG	0

EXAMPLE 4

Identification of specific epitopes:-

Many peptide fragments having the amino acid sequence of TNF indicated in Table 2 were synthesized using peptide synthesizer 430A of Applied Bio System Inc. on the basis of the amino acid sequence of a known TNF. After the synthesis, the peptide fragments were dissociated from a supporting resinous structure by treatment with hydrogen fluoride, and subjected to inverse-phase column chromatography to determine their purities and as required, purified. The peptide fragments having a sufficient degree of purification were used in the experiment.

Each purified peptide fragment (0.5 to 1 mg/ml) was allowed to stand overnight at 4 °C on an immunoassay plate (Titertek) and fixed. The plate was washed three times with a washing solution containing 0.5 % bovine serum albumin (20 mM phosphate buffer, 0.136M NaCl, 0.05 % Tween 20, 0.2 % NaN₃). Then, a buffer containing 1 % bovine serum albumin (20 mM phosphate buffer, 0.135M NaCl, 0.2 % NaN₃) was added, and the plate was left to stand at room temperature for 1 to 2 hours. The plate was then washed three times with the aforesaid washing solution. The purified anti-TNF antibody was added and reacted at room temperature for 1 to 2 hours. The plate was washed three times with the aforesaid washing solution, and goat-antimouse IgG-conjugated alkaline phosphatase was added, and the plate was further left to stand at room temperature for 1 to 2 hours. The plate was washed with the aforesaid washing solution and p-nitrophenyl phosphate, disodium, a substrate for the alkaline phosphatase, was added in a concentration of 1 mg/ml. Changes in absorbance per minute at a wavelength of 405 nm were measured by an ELISA analyzer (ETY-86 supplied by Toyo Sokki K. K.).

The reactivity of the antibodies with various peptides was examined by the above method, and the antibodies showed the binding activity shown in Table 3. It was found that the antibodies capable of neutralizing TNF activity specifically bind to a peptide having the following sequence which correspond to the 68th to the 97th of the amino acid sequence of TNF.

5 **NH₂-Gly Cys Pro Ser Thr Val Leu L u Thr**
 His Thr Ile Ser Arg Ile Ala Val S r Tyr Gln
 Thr Lys Val Asn Leu Leu Ser Ala Ile-COOH.

10 The three antibodies incapable of neutralizing the activity of TNF (1F12A7, 1F12A9, 9E8G7) bound to
 the synthetic peptide fragments D and J in Table 2, and thus specifically recognize epitopes contained in
 the 113th to the 127th amino acid in the amino acid sequence of human tumor necrosis factor and which is
 common to the peptides D and J.

15 **NH₂-Pro Trp Tyr Glu Pro Ile Tyr Leu Gly**
 Gly Val Phe Gln Leu Glu-COOH.

20 One antibody (1A10D4) incapable of neutralizing the activity of TNF specifically bound to a peptide
 having the following sequence corresponding to the 7th to the 37th amino acids in the amino acid sequence
 of TNF.

25 **NH₂-Thr Pro Ser Asp Lys Pro Val Ala His**
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln
 Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala
 Leu Leu-COOH.

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Table 2

Synthetic Peptides

- 5 A. H₂N-Thr Pro Ser Asp Lys Pro Val Ala His
Val Val Ala Asn Pro Gln Ala Glu Gly Gln
Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala
Leu Leu-COOH
- 10 B. H₂N-Ala Asn Gly Val Glu Leu Arg Asp Asn
Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu
Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln-COOH
- 15 C. H₂N-Gly Cys Pro Ser Thr His Val Leu Leu
Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr
Gln Thr Lys Val Asn Leu Leu Ser Ala Ile-COOH
- 20 D. H₂N-Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu
Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile
Tyr Leu Gly Gly Val Phe Gln Leu Glu-COOH
- 25 E. H₂N-Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn
Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly
Gln Val Tyr Phe Gly Ile Ile Ala Leu-COOH
- F. H₂N-Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
Leu Arg Asp Asn Gln Leu Val-COOH
- 30 G. H₂N-Gln Val Leu Phe Lys Gly Gln Gly Cys Pro
Ser Thr His Val-COOH
- H. H₂N-Ser Thr His Val Leu Leu Thr His Thr Ile
Ser Arg Ile Ala Val Ser-COOH
- 35 I. H₂N-Ile Ala Val Ser Tyr Gln Thr Lys Val Asn
Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg
Glu Thr Pro Glu Gly Ala Glu Ala Lys-COOH
- 40 J. H₂N-Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser
Ala Glu Ile Asn Arg Pro Asp Tyr Leu-COOH
- 45
- 50
- 55

Table 3
Binding of antibodies to synthetic peptides

	A	B	C	D	E	F	G	H	I	J
11D7G4	0.4	0.3	4.1	0.5	0.3	0.2	0.1	1.6	0.3	0.5
9C4G5	0.5	0.3	3.4	0.4	0.3	0.1	0.1	0.2	0.2	0.1
10B7E11	0.5	0.3	2.4	0.3	0.2	N.D.	0.1	N.D.	0.1	N.D.
8E6G4	0.3	0.2	0.8	0.2	0.1	N.D.	0.1	N.D.	0.1	N.D.
8E6B6	0.1	0.1	0.4	0.1	0.1	N.D.	N.D.	N.D.	N.D.	N.D.
9C4A9	0.2	0.1	0.5	0.2	0.0	N.D.	N.D.	N.D.	N.D.	N.D.
1F12A7	0.5	0.5	5.1	44.1	2.5	N.D.	7.9	11.1	6.5	86.4
1F12A9	2.0	0.3	1.3	34.3	2.7	N.D.	0.1	N.D.	0.2	N.D.
9E8G7	0.6	0.1	0.4	33.0	0.1	N.D.	N.D.	N.D.	N.D.	N.D.
1A10D4	28.6	0.7	1.9	4.8	0.4	N.D.	N.D.	N.D.	N.D.	N.D.

The numerals show the enzyme activity ($\Delta OD_{405}/\text{min}$) of the bound labelled antibodies. N.D. indicates that no measurement was done.

To determine the epitopes of TNF to which the antibodies were bound, the following experiment was conducted.

The purified peptide fragment (1 microgram/ml) was allowed to stand overnight at 4 °C on an immunoassay plate (Titertek) and fixed. The plate was washed three times with a washing solution

containing 0.5 % bovine serum albumin (20 mM phosphate buffer, 0.136M NaCl, 0.05 % Tween 20, 0.2 % NaN₃). Then, a buffer containing 1 % bovine serum albumin (20 mM phosphate buffer, 0.135M NaCl, 0.2 % NaN₃) was added, and the plate was left to stand at room temperature for 1 to 2 hours. The plate was then washed three times with the aforesaid washing solution. A mixture of the purified anti-TNF antibody and the synthetic peptide, which had been pre-incubated at 37 °C for 1 hour, was added and reacted at room temperature for 1 to 2 hours. The plate was washed three times with the aforesaid washing solution, and goat-anti-mouse IgG-conjugated alkaline phosphatase was added, and the plate was further left to stand at room temperature for 1 to 2 hours. The plate was washed with the aforesaid washing solution and p-nitrophenyl phosphate, disodium, a substrate for the alkaline phosphatase, was added in a concentration of 1 mg/ml. Changes in absorbance per minute at a wavelength of 405 nm were measured by an ELISA analyzer (ETY-86 supplied by Toyo Sokki K. K.).

Figure 2 shows that the synthetic peptide D (see Table 2) inhibits the binding of the monoclonal antibody 1F12A7 depending upon the dose. The ratio of the antibody bound to THF in the presence of the peptide is shown in Figure 2 with the amount of the antibody bound to TNF in the absence of the synthetic peptide being taken as 100 %. The results demonstrate that the monoclonal antibody 1F12A7 bound to a portion containing the 98th Lys to the 127th Glu of the amino acid sequence of TNF.

EXAMPLE 5

Effect of antibodies on the binding of TNF to TNF receptor:-

Using TNF having ¹²⁵I introduced therein by enzymebeads (Bio-Rad) (specific radioactivity 1.2x10¹⁰ cpm/mg protein), the effect of the anti-TNF antibody on the binding of ¹²⁵I-TNF to the TNF receptor on L929 cells. The L929 cells (3x10⁶ cells) were seeded in a dish and incubated at 37 °C for 4 hours. The supernatant was discarded, and a sample obtained by pre-incubating ¹²⁵I-TNF (51 ng/ml) or ¹²⁵I-TNF (51 ng/ml) and anti-TNF antibody (5.1 micrograms/ml) at room temperature for 1 hour was added to the cells. On the other hand, to determine the specific binding to the receptor, non-labelled TNF was added to the sample in an amount 100 times as large as the sample was added to the L929 cells, and the mixture was incubated at 37 °C for 2 hours. The cells were collected by a scraper and washed twice with a culture medium (Eagle medium containing 5 % bovine fetal serum) by a centrifuging operation. Then, the radioactivity of ¹²⁵I contained in the cells was measured. As an example of the results, Table 4 below shows the results obtained when anti-TNF antibody 11D7G4 capable of neutralizing the activity of TNF was used. The results clearly show that the anti-TNF antibody 11D7G4 specifically inhibits the binding of TNF to the TNF receptor.

Table 4 also show the results obtained with 1F12A7 incapable of neutralizing the activity of TNF and 10B7E11 capable of inhibiting the binding of TNF to the TNF receptor. These results demonstrate that the anti-TNF antibody 1F12A7 which recognizes epitopes including the 113th to the 127th amino acids of the amino acid sequence of TNF does not at all inhibit the binding of TNF to the TNF receptor.

Table 4

Treatment	Count (cpm) of the specific binding to L929 cells
^{125}I -TNF (51 ng/ml)	844.0 \pm 151.0
^{125}I -TNF (51 ng/ml) + 11D7G4 (5.1 g/ml)	0 \pm 48.2
^{125}I -TNF (51 ng/ml) + 10B7E11 (5.1 g/ml)	-18.5 \pm 155.6
^{125}I -TNF (51 ng/ml) + 1F12A7 (5.1 g/ml)	860 \pm 24.4
^{125}I -TNF (51 ng/ml) + mouse IgG (5.1 g/ml)	837.2 \pm 72.2

EXAMPLE 6

Preparation of a calibration curve:-

The monoclonal antibody "11D7G4" produced in Example 1 was fixed to an insoluble carrier (immunoassay plate) as shown below and used as an antibody in this example. The monoclonal antibody "9C4G5" was labelled with alkaline phosphatase (a product of Sigma Co.) derived from bovine pancreas and used as a second antibody.

Monoclonal antibody (11D7G4) in a concentration of 15 micrograms/ml was left to stand overnight at 4 °C on an immunoassay plate (Titertek) and fixed. The plate was washed three times with a washing solution containing 0.5 % bovine serum albumin (20 mM phosphate buffer, 0.135M NaCl, 0.05 % Tween 20, 0.2 % NaN₃). Then, a buffer containing 1 % bovine serum albumin (20 mM phosphate buffer, 0.135M NaCl, 0.2 % NaN₃) was added, and the plate was left to stand at room temperature for 1 hour. It was washed three times with the above washing solution, and then human TNF in various concentrations was added and reacted at room temperature for 1 hour. The plate was washed three times with the above washing solution, and the second antibody (9C4G5) labelled with alkaline phosphatase was added and reacted at room temperature for 1 hour. The plate was washed with the above washing solution, and p-nitrophenyl

phosphate, disodium, a substrate for the alkaline phosphatase, was added in a concentration of 1 mg/ml. Changes in absorbance per minute at a wavelength of 405 nm were measured by an ELIZA analyzer (ETY-96). The results are shown in Figure 3. From this figure, the amount of human TNF can be easily measured by enzyme-linked immunosorbent assay using a monoclonal antibody, which specifically recognizes human TNF, in accordance with the sandwich technique.

EXAMPLE 7

Measurement of TNF in the serum of a Kawasaki disease patient:-

Serum taken from a patient of Kawasaki disease, and as controls the serum of a normal healthy person and the serum of a patient of another disease were each diluted to 2 to 10 times with a phosphate buffer (20 mM phosphate buffer, 0.135M NaCl, pH 7.2) and used as antigens. The amount of TNF in these samples was measured by the sandwich method described in Example 6. Calibration curves used were prepared simultaneously under the same conditions.

The results are shown in Figure 4. TNF was detected from the serum samples of 21 patients (40 %) out of 30 patients. As shown in Figure 5, the detection of TNF is significantly related to the occurrence of a diseased change in coronary artery. As Figure 6 shows, TNF was well detected in the acute stage of a patient with great severity.

Measurement of the amount of TNF by using the detection kit of this invention is very effective for diagnosing Kawasaki disease and analyzing its condition, especially for the anticipatory diagnosis of the occurrence of a diseased condition in the coronary artery.

Claims

1. A method for confirming a diagnosis of Kawasaki disease in a patient which comprises
 - contacting an anti-human tumor necrosis factor antibody with a sample of body fluid from the patient,
 - detecting an amount of a substance which specifically binds the anti-human tumor necrosis factor antibody, and
 - comparing the amount of the substance with that in a sample of body fluid taken from a normal, healthy person
 wherein elevated levels of the substance are indicative of Kawasaki disease.
2. The method of claim 1 wherein the anti-human tumor necrosis factor antibody is an anti-human tumor necrosis factor monoclonal antibody.
3. The method of claim 2 wherein the anti-human tumor necrosis factor monoclonal antibody is characterized by
 - (a) having the ability to neutralize the cytotoxic effect of human tumor necrosis factor on L929 cells and the inhibitory effect of human tumor necrosis factor on fatty acid metabolism,
 - (b) recognizing epitopes contained in the 68th (Gly) to the 97th (Ile) amino acids in the amino acid sequence of human tumor necrosis factor, and
 - (c) specifically inhibiting binding of human tumor necrosis factor to a tumor necrosis factor receptor.
4. The method of claim 1 comprising
 - (a) contacting the body fluid sample with a first anti-human tumor necrosis factor antibody immobilized on a solid support, then contacting the solid support with a labelled second anti-human tumor necrosis factor antibody, or
 - (b) contacting the immobilized first anti-human tumor necrosis factor antibody, the labelled second anti-human tumor necrosis factor antibody and the body fluid sample simultaneously, and
 - detecting the amount of the substance which specifically binds anti-human tumor necrosis factor antibody by detecting the amount of bound labelled antibody.
5. The method of claim 1 wherein the body fluid is serum or plasma taken from the patient.
6. A detection kit for confirming a diagnosis of Kawasaki disease of a patient by detecting the amount of a substance which is specifically bound to the anti-human tumor necrosis factor antibody in a body fluid

taken from a patient, said kit comprising a first anti-human tumor necrosis factor antibody, a second anti-human tumor necrosis factor antibody and an insoluble solid carrier, one of the first and second antibodies being fixed to the solid carrier.

- 5 7. The detection kit of claim 6 wherein at least one of the first and second antibodies is an anti-human tumor necrosis factor monoclonal antibody.
8. The detection kit of claim 6 wherein at least one of the first and second antibodies is an anti-human tumor necrosis factor monoclonal antibody which can neutralize the cytotoxic effect of human tumor
10 necrosis factor on L929 cells.

Patentansprüche

1. Verfahren zur Bestätigung einer Diagnose der Kawasaki-Krankheit bei einem Patienten, dadurch
15 **gekennzeichnet**, daß
ein anti-humane Tumor-Nekrosefaktor-Antikörper mit einer Probe der Körperflüssigkeit des Patienten behandelt wird,
eine Menge einer Substanz, die spezifisch den anti-humanen Tumor-Nekrosefaktor-Antikörper bindet, nachgewiesen wird und
20 die Menge der Substanz mit der in einer Probe einer Körperflüssigkeit, die einem normalen gesunden Menschen entnommen worden ist, verglichen wird,
wobei erhöhte Gehalte der Substanz ein Anzeichen für die Kawasaki-Krankheit sind.
2. Verfahren nach Anspruch 1, dadurch **gekennzeichnet**, daß der anti-humane Tumor-Nekrosefaktor-Antikörper ein monoklonaler anti-humane Tumor-Nekrosefaktor-Antikörper ist.
25
3. Verfahren nach Anspruch 2, dadurch **gekennzeichnet**, daß der monoklonale anti-humane Tumor-Nekrosefaktor-Antikörper charakterisiert ist durch
30 (a) die Fähigkeit, die cytotoxische Wirkung von humanem Tumor-Nekrosefaktor auf L929-Zellen zu neutralisieren, und eine Inhibitorwirkung auf den humanen Tumor-Nekrosefaktor im Fettsäure-Metabolismus,
(b) Erkennung der Epitope, die in den 68. (Gly) bis 97. (Ile) Aminosäuren in der Aminosäuresequenz des humanen Tumor-Nekrosefaktors vorkommen, und
(c) spezifische Inhibierung der Bindung des humanen Tumor-Nekrosefaktors an einen Tumor-Nekrosefaktor-Rezeptor.
35
4. Verfahren nach Anspruch 1, dadurch **gekennzeichnet**, daß
(a) die Körperflüssigkeitsprobe mit einem ersten anti-humanen Tumor-Nekrosefaktor-Antikörper, der auf einem festen Träger immobilisiert ist, behandelt wird, dann der feste Träger mit einem
40 markierten zweiten anti-humanen Tumor-Nekrosefaktor-Antikörper in Kontakt gebracht wird oder
(b) der immobilisierte erste anti-humane Tumor-Nekrosefaktor-Antikörper, der markierte zweite anti-humane Tumor-Nekrosefaktor-Antikörper und die Körperflüssigkeitsprobe gleichzeitig in Kontakt gebracht werden und
die Menge der Substanz, die spezifisch an den anti-humanen Tumor-Nekrosefaktor-Antikörper
45 bindet, nachgewiesen wird, indem die Menge des gebundenen markierten Antikörpers nachgewiesen wird.
5. Verfahren nach Anspruch 1, dadurch **gekennzeichnet**, daß die Körperflüssigkeit Serum oder Plasma, das dem Patienten entnommen wurde, ist.
50
6. Nachweiskit für die Bestätigung einer Diagnose der Kawasaki-Krankheit eines Patienten durch Nachweis der Menge einer Substanz, die spezifisch an den anti-humanen Tumor-Nekrosefaktor-Antikörper in einer Körperflüssigkeit, die einem Patienten entnommen worden ist, gebunden ist, wobei das Kit einen ersten anti-humanen Tumor-Nekrosefaktor-Antikörper, einen zweiten anti-humanen Tumor-Nekrosefaktor-Antikörper und einen unlöslichen festen Träger umfaßt, wobei entweder der erste oder der zweite
55 Antikörper an einen festen Träger fixiert ist.

7. Nachweiskit nach Anspruch 6, dadurch **gekennzeichnet**, daß mindestens entweder der erste oder der zweite Antikörper ein monoklonaler anti-humaner Tumor-Nekrosefaktor-Antikörper ist.
8. Nachweiskit nach Anspruch 6, dadurch **gekennzeichnet**, daß mindestens entweder der erste oder der zweite Antikörper ein monoklonaler anti-humaner Tumor-Nekrosefaktor-Antikörper ist, der die cytotoxische Wirkung von humanem Tumor-Nekrosefaktor auf L929-Zellen neutralisieren kann.

Revendications

1. Procédé pour confirmer le diagnostic de la maladie de Kawasaki chez un patient qui comprend
 - la mise en contact d'un anticorps anti-facteur de nécrose tumorale humain avec un échantillon de liquide corporel prélevé sur le malade,
 - la détection de la quantité de substance qui se lie spécifiquement à l'anticorps anti-facteur de nécrose tumorale humain, et
 - la comparaison de la quantité de cette substance avec celle d'un échantillon de fluide corporel prélevé sur un individu normal, en bonne santé, procédé dans lequel des taux élevés de cette substance indiquent une atteinte par la maladie de Kawasaki.
2. Procédé selon la revendication 1 dans lequel l'anticorps anti-facteur de nécrose tumorale humain est un anticorps monoclonal dirigé contre le facteur de nécrose tumorale humain.
3. Procédé selon la revendication 2 dans lequel l'anticorps monoclonal anti-facteur de nécrose tumorale humain est caractérisé en ce qu'il
 - (a) a le pouvoir de neutraliser l'effet cytotoxique du facteur de nécrose tumorale humain sur les cellules L929 ainsi que l'effet inhibiteur de ce facteur sur le métabolisme des acides gras,
 - (b) reconnaît les épitopes situés entre le 68^{ème} (Gly) et le 97^{ème} (Ile) acide aminé de la séquence d'acides aminés du facteur de nécrose tumorale humain, et
 - (c) inhibe de manière spécifique la liaison du facteur de nécrose tumorale humain à son récepteur.
4. Procédé selon la revendication 1 comprenant les étapes suivantes:
 - (a) mettre l'échantillon de fluide corporel en contact avec un premier anticorps anti-facteur de nécrose tumorale humain immobilisé sur un support solide, puis à amener le support solide en contact avec un second anticorps anti-facteur de nécrose tumorale humain marqué, ou à
 - (b) mettre l'anticorps anti-facteur de nécrose tumorale humain immobilisé, le second anticorps anti-facteur de nécrose tumorale humain marqué et l'échantillon de fluide corporel en contact simultanément, et
 - (c) à doser la quantité de substance qui se lie spécifiquement à l'anticorps anti-facteur de nécrose tumorale humain en quantifiant l'anticorps marqué fixé.
5. Procédé selon la revendication 1 dans lequel l'échantillon de fluide corporel est du sérum ou du plasma prélevé sur le malade.
6. Kit de détection pour confirmer le diagnostic de la maladie de Kawasaki chez un patient en quantifiant la substance contenue dans un fluide corporel prélevé sur le malade qui se lie spécifiquement à l'anticorps anti-facteur de nécrose tumorale humain, ledit kit comprenant un premier anticorps anti-facteur de nécrose tumorale humain, un second anticorps anti-facteur de nécrose tumorale humain et un support solide insoluble, l'un des premier et second anticorps étant attaché au support solide.
7. Kit de détection selon la revendication 6 dans lequel l'un au moins des premier et second anticorps est un anticorps monoclonal anti-facteur de nécrose tumorale humain.
8. Kit de détection selon la revendication 6 dans lequel l'un au moins des premier et second anticorps est un anticorps monoclonal anti-facteur de nécrose tumorale humain capable de neutraliser l'effet cytotoxique de ce facteur sur les cellules L929.

FIG. 1

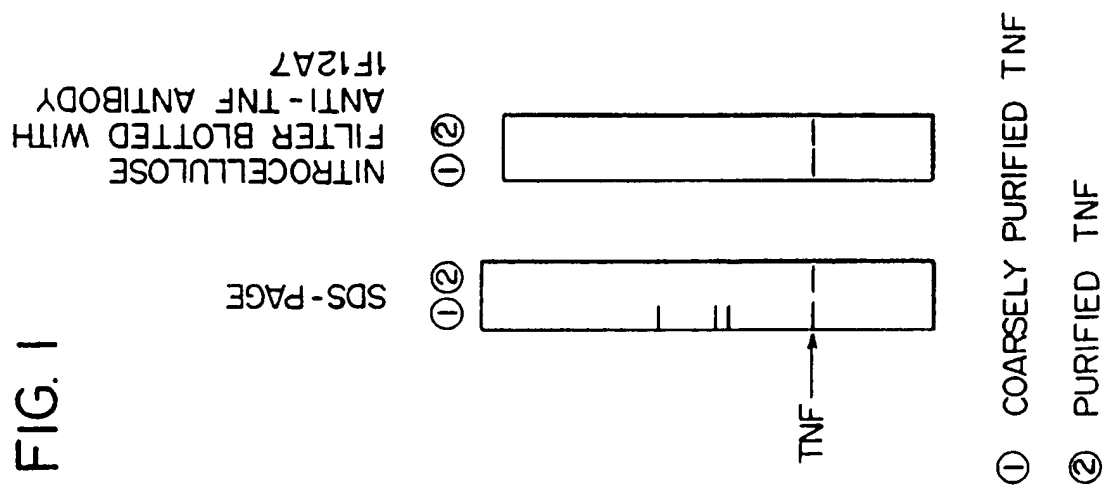


FIG. 2

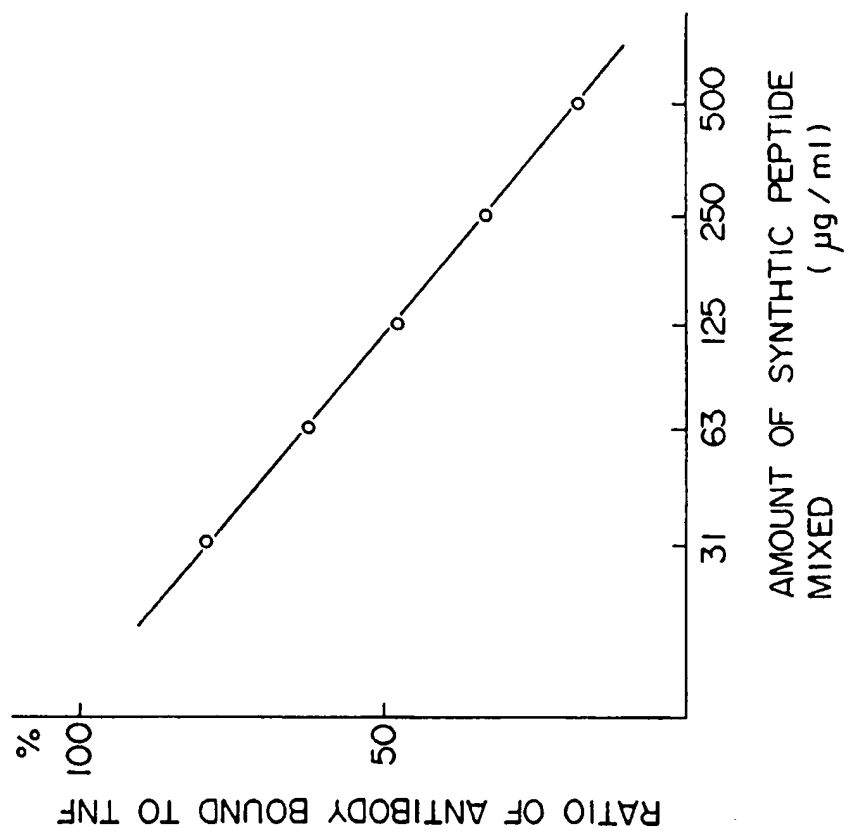


FIG. 3

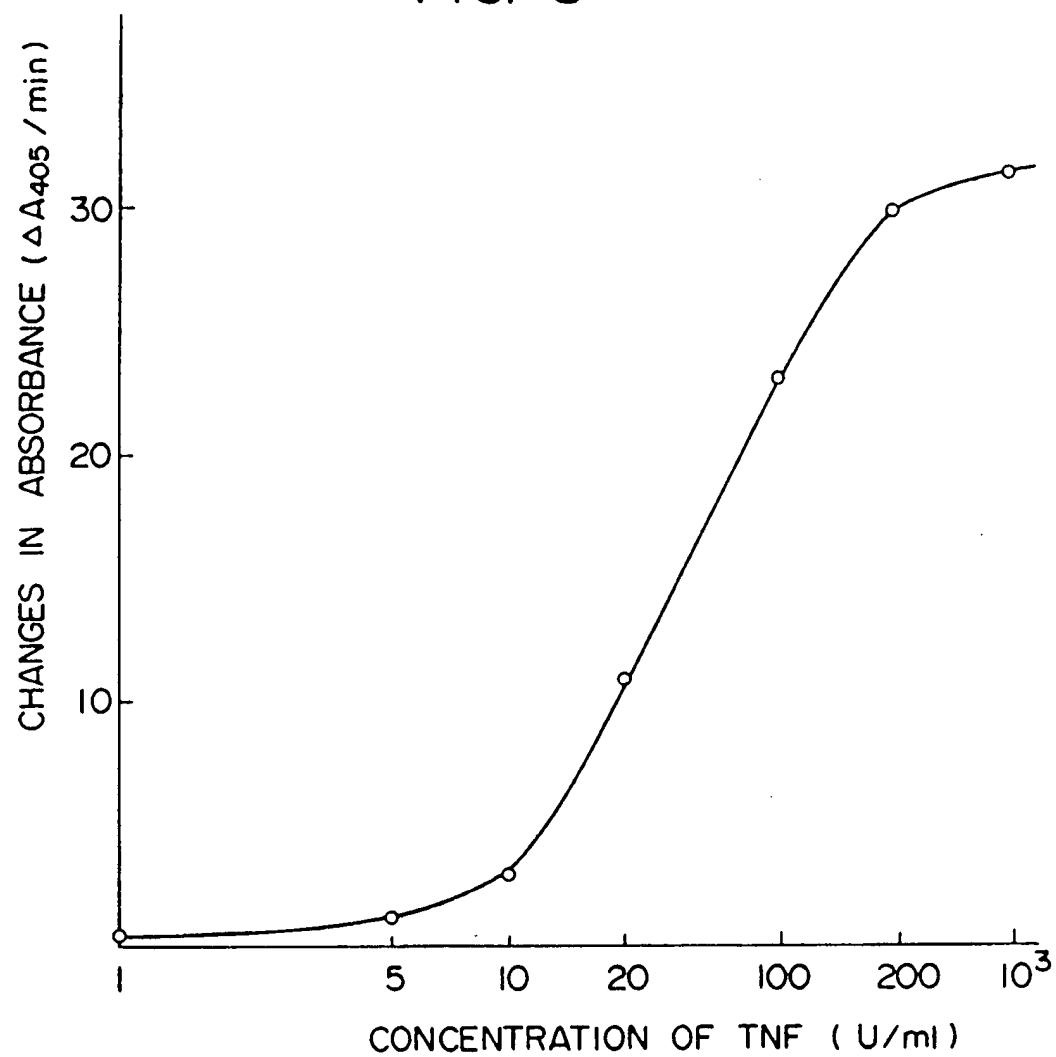


FIG. 4

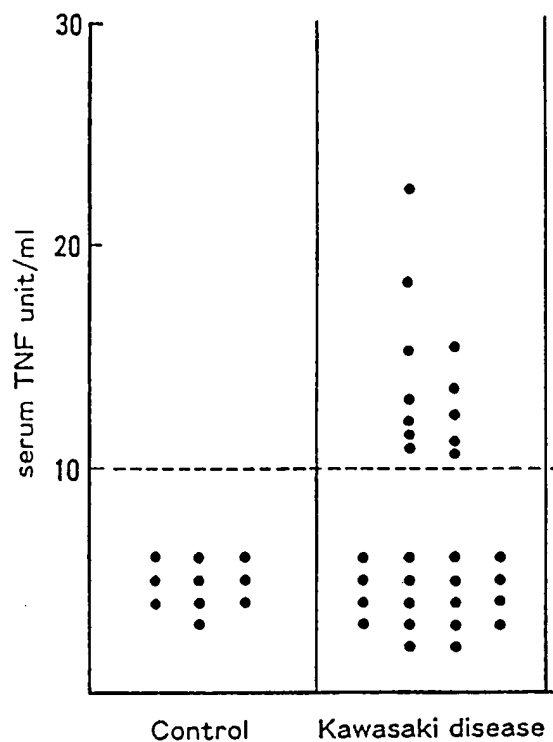


FIG. 5

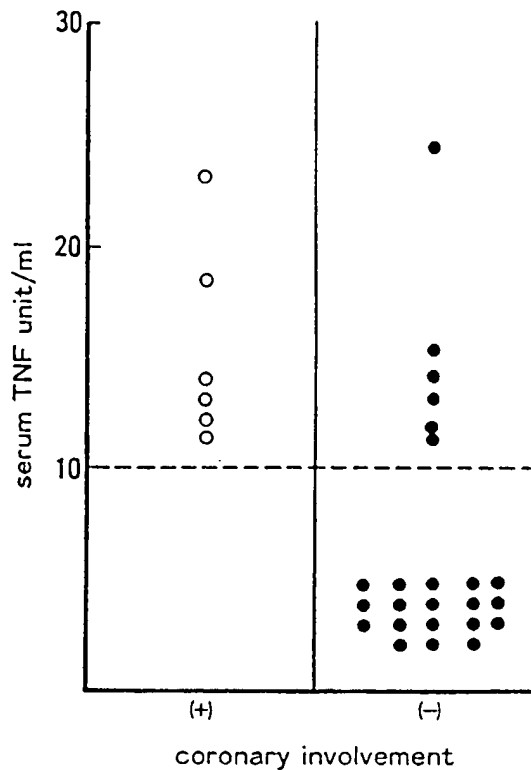
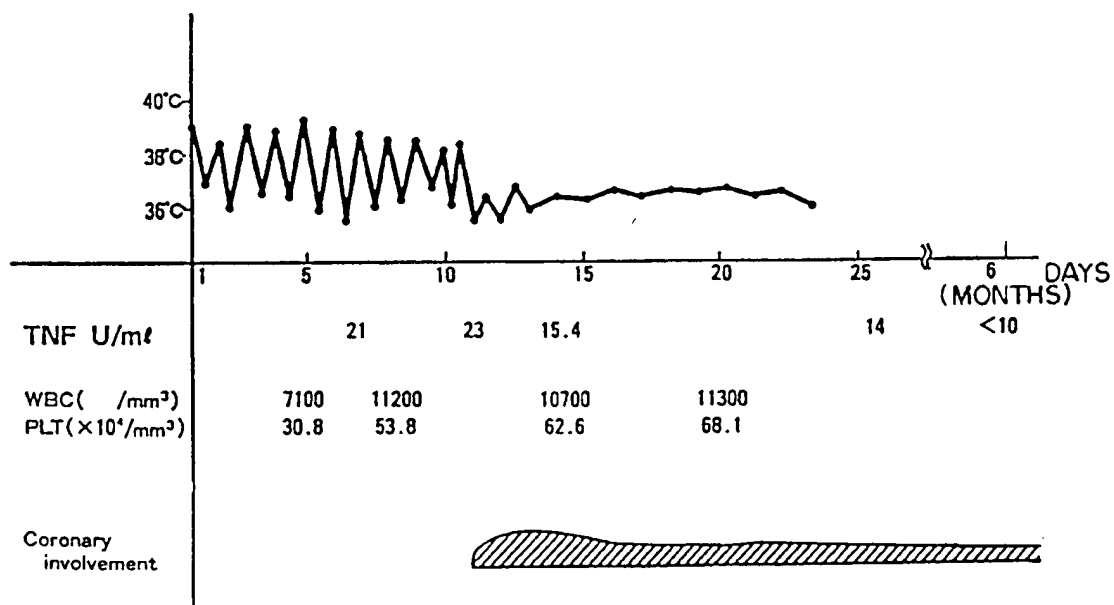


FIG. 6



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(54) Preparation of pure human tumor necrosis factor and hybridomas producing monoclonal antibodies to human tumor necrosis factor.

(57) Human TNF(LuKII) having a specific activity of at least 1.5×10^5 units per milligram of total protein is produced by contacting a TNF-containing protein composition, which has been harvested from human cell lines of hematopoietic origin or recombinant origin, in separate adsorption stages with glass

beads, lentil lectin bound to Sepharose, and procion red agarose, thereby selectively to adsorb TNF in each stage, while leaving some impurities unadsorbed, each contact stage being followed by contact of the adsorbent with an eluant thereby to obtain a solution of more highly purified TNF after each

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stage. The purified human TNF(LuKII) is used to produce monoclonal antibodies against TNF(LuKII) and such antibodies can be used to assay samples for the presence of TNF(LuKII).



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Application number

EP 86 11 1926

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 105, no. 11, September 15, 1986 page 493, abstract 95800j Columbus, Ohio, US B.Y. RUBIN et al.: "Human tumor necrosis factor (LuKII): recent developments" & ADV. IMMUNOPHARMACOL.3, Proc. Int. Conf., 3rd, 1985 (publ. 1986), 139-48 * Abstract *	1-14	C 12 P 21/02 C 12 N 5/00 C 12 P 21/00 G 01 N 33/577 A 61 K 37/24
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 260, no. 4, February 25, 1985, pages 2345-2354 B.B. AGGARWAL et al. "Human tumor necrosis factor; production, purification, and characterization" * Whole document *	1-6	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 105; no. 23, December 8, 1986, ./.		A 61 K C 12 P
INCOMPLETE SEARCH <p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.</p> <p>Claims searched completely: 1-19 Claims searched incompletely: Claims not searched: 20 Reason for the limitation of the search:</p> <p>Method for treatment of the human or animal body by surgery or therapy (See art. 52(4) of the European Patent Convention)</p>			
Place of search The Hague		Date of completion of the search 22-12-1988	Examiner CHARLES
CATEGORY OF CITED DOCUMENTS <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			



DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
	page 451, abstract 207220d Columbus, Ohio, US H.HAYASHI et al. "An enzyme-linked immunosorbent assay for recombinant human tumor necrosis factor using monoclonal antibody" * Abstract *	15-19	
	--		
X,P	PROC. NATL. ACAD. SCI., USA, vol. 82, October 1985, pages 6637-6641 B.Y. RUBIN et al.: "Purification and characterization of a human tumor necrosis factor from the LuKII cell line" * Whole document *	1-19	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
	--		
X,P	CHEMICAL ABSTRACTS, vol. 104, no. 11, March 17, 1986, page 534, abstract 86989e Columbus, Ohio, US & JP-A-60 208 924 (ASAHI CHEMICAL INDUSTRY CO., LTD.). 21-10-1985 * Abstract *	15-19	
	--		
Y,P	EP-A-0 168 214 (GENENTECH., INC.) * Page 5, lines 20-25; page 9, lines 5-15; page 28, line 12 - page 29, line 26; claims 1-7 *	13,14	
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Y,P	EP-A-0 183 198 (ASAHI KASEI KOGYO KABUSHIKI KAISHA) * Page 5, line 16 - page 6, line 8; page 7, line 13 - page 8, line 4; page 21, lines 6-21; claims 1,4 *	13,14	
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Y,P	DE-A- 3 421 731 (BOEHRINGER INGELHEIM INTERNATIONAL GmbH) * Page 10, paragraph 1; page 12, paragraph 2; page 20, point 2; page 23, point 4; claims 1-3 *	13,14	
